INCORPORATION OF MEMBRANE PROTEINS INTO LARGE SINGLE BILAYER VESICLES

Application to Rhodopsin

A. DARSZON, C. A. VANDENBERG, M. H. ELLISMAN, and M. MONTAL. From the Departments of Biology, Physics, and Neurosciences, University of California, San Diego, La Jolla, California 92093

ABSTRACT

A general procedure to incorporate membrane proteins in a native state into large single bilayer vesicles is described. The results obtained with rhodopsin from vertebrate and invertebrate retinas are presented. The technique involves: (*a*) the direct transfer of rhodopsin-lipid complexes from native membranes into ether or pentane, and (*b*) the sonication of the complex in apolar solvent with aqueous buffer followed by solvent evaporation under reduced pressure. The spectral properties of rhodopsin in the large vesicles are similar to those of rhodopsin in photoreceptors; furthermore, bleached bovine rhodopsin is chemically regenerable with 9-cis retinal. These results establish the presence of photochemically functional rhodopsin in the large vesicles. Freeze-fracture replicas of the vesicles reveal that both internal and external leaflets contain numerous particles ~80 Å in diameter, indicating that rhodopsin is symmetrically distributed within the bilayer. More than 75% of the membrane area is incorporated into vesicles larger than 0.5 μm and ~40% into vesicles larger than 1 μm.

KEY WORDS membranes · liposomes · rhodopsin · reconstitution · freeze-fracture

Lipid bilayer vesicles, or liposomes, have been extensively studied in recent years as membrane models. They have been particularly useful in functional reconstitution studies since they can be simultaneously reassembled with membrane proteins to yield vesicles which exhibit biological activity (for recent reviews, see references 11, 15, and 21).

Bilayer vesicles have the disadvantage of a very small inner volume (5, 19) restricting the ability to entrap large markers or to study the electrical properties with microelectrode techniques. This problem was overcome when cell-size single bilayer vesicles were formed by the sequential hydration and dehydration of lipid samples; they were reported to have diameters ranging from 0.5 to 1.0 μm (22, 23) and up to 300 μm (1, 17), and not to form in electrolyte solutions or in the presence of proteins (22, 23). At present, however, the adequacy of this procedure to incorporate native membrane proteins into large vesicles appears promising (1).

Szoka and Papahadjopoulos (25) recently reported an elegant method to form large unilamellar vesicles by sonicating a phospholipid solution in an organic solvent with an aqueous buffer followed by solvent evaporation in a rotatory evaporator under reduced pressure. These vesicles entrap a large percentage of the aqueous phase (15–65%) including large macromolecules. This approach appears particularly suitable to incorporate membrane proteins into large vesicles using the procedures we have developed to extract protein-lipid complexes of membrane proteins into apolar solvents. In these extracts the protein retains its biological activity in the solvent or after its removal (3, 4, 14).

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/79/05/0446/07 $1.00
Volume 81 May 1979 446-452

Here, we combine these two approaches and report the formation of large single bilayer vesicles containing photochemically active rhodopsin. The properties of the preparation and the general application of the technique developed to membrane reconstitution studies are discussed.

MATERIALS AND METHODS

Materials

The following materials were obtained from the indicated sources: dark-adapted bovine retinas (Hormel Co., Austin, Minn.); dark-adapted squid retinas (dissected from live *Loligo opalescens*, Sea Life Supply, Monterey, Calif.); L-α-lecithin from soybeans and 9-cis retinal (Sigma Chemical Co., St. Louis, Mo.); spectroquality *n*-pentane (Matheson Scientific, Elk Grove, Ill.) and diethyl ether (Mallinckrodt Inc., St. Louis, Mo.). All other reagents were of the highest purity commercially available. Glass-redistilled water was used throughout.

Preparative Procedures

MEMBRANE PREPARATIONS: All the procedures were performed under dim red light at 4°C unless otherwise specified. Rod outer segments (ROS) from dark-adapted cattle retinas were isolated by sucrose flotation and purified in a discontinuous gradient (13, 20). ROS membrane aliquots in 10 mM Tris-acetate buffer, pH 7.4, containing ~0.75 mg of rhodopsin were centrifuged at 27,000 *g* for 20 min, the supernate was removed, and the pellet was frozen in liquid nitrogen for storage at −70°C. Squid rhabdomere outer segments from dark-adapted retinas were isolated and purified as follows: 40 thawed retinas were shaken in 0.4 M Na phosphate, 2 mM EDTA, pH 6.8, and the crude outer segments were centrifuged at 35,000 *g* for 20 min. The pellet was homogenized in 40% (wt/vol) sucrose in buffer (0.3 M NaCl, 0.1 M Na phosphate, 2 mM EDTA, pH 6.8) and layered under a discontinuous density gradient of 33% (wt/vol) sucrose in buffer and of buffer only. This was centrifuged in a Beckman SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 22,000 rpm for 1 h. The material floating at the 33%/buffer interface was collected, washed twice (27,000 *g* for 30 min) in 10 mM imidazole, 1 mM EDTA, pH 7.0, and the pelleted membranes were frozen in liquid nitrogen for storage at −70°C.

DIRECT TRANSFER OF RHODOPSIN FROM PURIFIED RETINAL PHOTORECEPTOR MEMBRANES INTO APOLAR SOLVENTS (3): An ROS pellet containing 0.75 mg of rhodopsin was resuspended in 1 ml of ether (or pentane) containing 50 mg of partially purified soybean phospholipids (12). The resulting ROS dispersion in ether was immediately sonicated for 6 min in a water bath sonicator (Bransonic, Heat Systems Ultrasonics, Plainview, N. Y., power output 100 W). Thereafter, 0.1 ml of 1 M $CaCl_2$ was added and the mixture was stirred in a vortex mixer for 2.5 min. The preparation was centrifuged in a clinical centrifuge (1 min), the ether phase was removed and diluted threefold with ether for vesicle formation.

Squid membranes were extracted following the same protocol with the following modifications: the pellet contained an absorbance of 0.90 o.d. at 490 nm determined after solubilizing an aliquot in 30 mM octylglucoside (24), 10 mM imidazole, 1 mM EDTA, pH 7.0; the phospholipid concentration was 25 mg in 1 ml of pentane; sonication time, 10 min; 0.05 ml of 1 M $CaCl_2$; vortex time, 5 min; centrifugation for 4 min at maximum speed. Two extracts were combined and diluted to 10 ml with pentane for vesicle formation.

FORMATION OF LARGE SINGLE BILAYER VESICLES CONTAINING RHODOPSIN: The ether extract containing bovine rhodopsin (3 ml) was mixed with the aqueous buffer (20 mM NaCl, 10 mM imidazole, pH 7.0) (1 ml), and the resulting two-phase system was sonicated for 4 min maintaining the system under argon. The mixture was then transferred to the rotary evaporator, and ether was removed under reduced pressure using the house vacuum (350 mm mercury). The 50-ml round-bottom flask was kept in contact with air at room temperature (20°C). A dry ice-acetone slurry was used in the condenser.

The pentane extract containing squid rhodopsin was mixed with the aqueous buffer at a volume ratio of 20:1 pentane:water and thereafter, sonicated, and evaporated as described before.

When the majority of the solvent was removed, a viscous gel formed which later became an aqueous suspension. Thereafter, an additional 0.5 ml of aqueous buffer was added and the suspension evaporated until no solvent was detected by olfactory analysis. The time required for solvent evaporation was ~1 h for ether and ~2 h for pentane. Vesicles containing only phospholipids were prepared following the same protocol.

Assay Procedures

Absorption spectra were recorded with a Cary 14 spectrophotometer (Applied Physics Corp., Monrovia, Calif.) on line with a minicomputer (2) and with a Perkin-Elmer model 555 spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.).

The regeneration of bovine rhodopsin (9) in the vesicles was assayed as follows: The vesicles were incubated in the dark with a 10-fold excess (molar basis) of 9-cis retinal. The membranes were bleached with 500 nm light (through a narrow band interference filter) and difference spectra recorded as a function of time.

Electron Microscope Preparations

FREEZE-FRACTURE: Vesicle preparations were glycerinated by pipetting one droplet of 100% glycerol into five droplets of experimental vesicle solutions. Droplets of these preparations were created on gold-

welled specimen supports (Balzers Corp., Nashua, N. H.), then frozen in Freon 22 held near its freezing point by liquid nitrogen. Fracturing was performed within a Balzers 301 freeze-etch device at −110°C and shadowed with the aid of an electronically coupled shuttering device (7). Replicas were floated on water, picked up on copper grids, and then observed in a JEOL 100 CX electron microscope operated at 80 kV.

NEGATIVE STAINING: Negatively stained samples (18) were prepared with 1% phosphotungstic acid at pH 7.0, then dried on a Formvar-filmed copper grid. The negatively stained preparations were used for vesicle size determination.

RESULTS AND DISCUSSION

The dark absorption spectrum of bovine rhodopsin incorporated in the large vesicles exhibits the characteristic maximum absorbance which disappears upon illumination. The difference spectrum, dark-bleached, shows a symmetric peak centered at ~505 nm (Fig. 1*A*, curve 1). The recovery of rhodopsin in the large vesicles is better than 80% of the original concentration present in the ether extract (0.5 ± 0.05 mg). This indicates that virtually all the protein is incorporated into the vesicles without inducing deleterious effects on the rhodopsin photochemical activity. Furthermore, a very sensitive criterion to evaluate the native state of rhodopsin is the ability of the bleached protein moiety opsin to combine with 9-cis retinal to regenerate isorhodopsin (9). Rhodopsin incorporated in the large vesicles is indeed regenerable. The extent of regeneration is 80% ± 9 (n = 3) (Fig. 1*A*, curves 2–4).

Irradiation of the vesicles containing squid rhodopsin with 500 nm light generates a mixture of two photoproducts, metarhodopsin$_{acid}$ (wavelength of maximum absorbance [λ_{max}] = 500 nm) and metarhodopsin$_{basic}$ (λ_{max} = 380 nm), which are in a pH-dependent equilibrium (8). At pH 11.0, the light-induced difference spectrum has a λ_{max} at ~490 nm (Fig. 1*B*, curve 1). Changing the pH to 7.2 leads to the conversion of metarhodopsin$_{basic}$ to metarhodopsin$_{acid}$ (8). A difference spectrum between the curves recorded initially at pH 11.0 and then at pH 7.2 exhibits a λ_{max} at ~500 nm with a recovery of absorbance in the visible (Fig. 1*B*, curve 2).

It is evident, therefore, that rhodopsin has not undergone major photochemical changes when extracted into apolar solvents and subsequently incorporated into large vesicles in aqueous phases, as described. This validates the notion that the full cycle of transferring a membrane protein from the native membrane into apolar media and back into a model membrane in aqueous solutions can be accomplished maintaining the biological activity of the protein (3, 4). The procedure described here is also applicable to protein-lipid complexes in apolar solvents derived from solubilized and purified membrane proteins (3, 14).

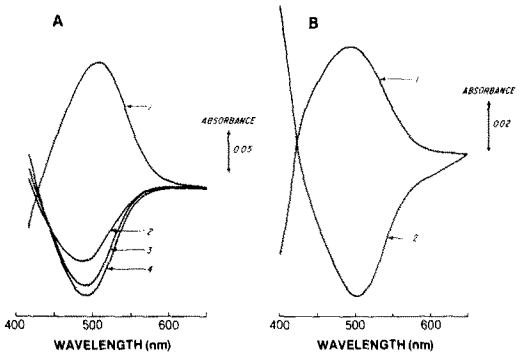

FIGURE 1 Absorption spectra of bovine and squid rhodopsin incorporated into large bilayer vesicles. (*A*) Bovine rhodopsin vesicles were prepared according to Materials and Methods. An aliquot (0.4 ml) was supplemented with 9-cis retinal in the dark (10-fold molar ratio with respect to rhodopsin). The dark spectrum was recorded and stored in the computer memory; thereafter, the sample was completely bleached (through a 500-nm interference filter) and the spectrum measured. Curve 1 represents the difference spectrum between the dark and bleached conditions. After bleaching, the sample was kept in the dark and spectra recorded after 31, 63, and 90 min. Curves 2, 3, and 4 represent the difference spectra between the bleached sample and these dark incubation periods, respectively. Temperature, 20°C. (*B*) Squid rhodopsin vesicles formed as described in Materials and Methods were buffered to pH 11.0 with Na_2CO_3. The medium had a final concentration of 23 mM Na_2CO_3, 10 mM hydroxylamine, 10 mM NaCl, 5 mM imidazole-HCl. Curve 1 is the difference spectrum between the dark and illuminated vesicles (total bleaching was performed through a 497-nm narrow band interference filter). The pH was then changed to 7.2 with 1 M HCl. Curve 2 is the difference spectrum of the bleached membranes between pH 11.0 and 7.2. Temperature, 0°C.

Critical aspects in the formation of the vesicles are the selection of the apolar solvent to be used as well as the volume ratio of aqueous to nonaqueous phases. The nature of the solvent is determined by the optimum partition of the protein-lipid complex ensemble into the solvent as judged by the extraction yield and the preservation of biological activity. The volume ratio (polar/apo-

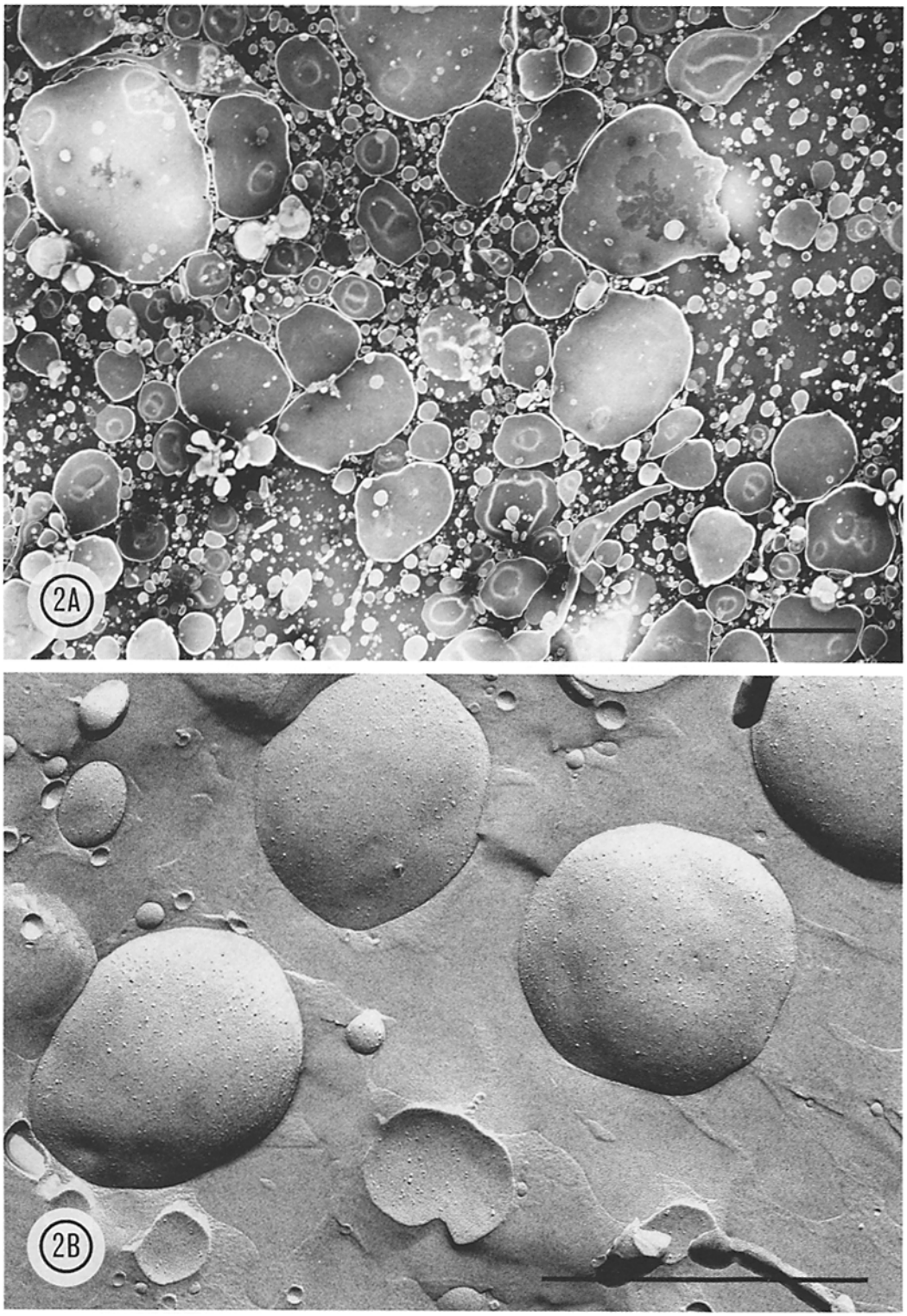

FIGURE 2 (*A*) Electron micrograph of negatively stained bilayer vesicles containing bovine rhodopsin. Bar, 1 μm. × 15,000. (*B*) Freeze-fracture appearance of the large bilayer vesicles containing bovine rhodopsin. Note that numerous particles are evenly distributed between the two fracture faces. Bar, 1 μm. × 50,000.

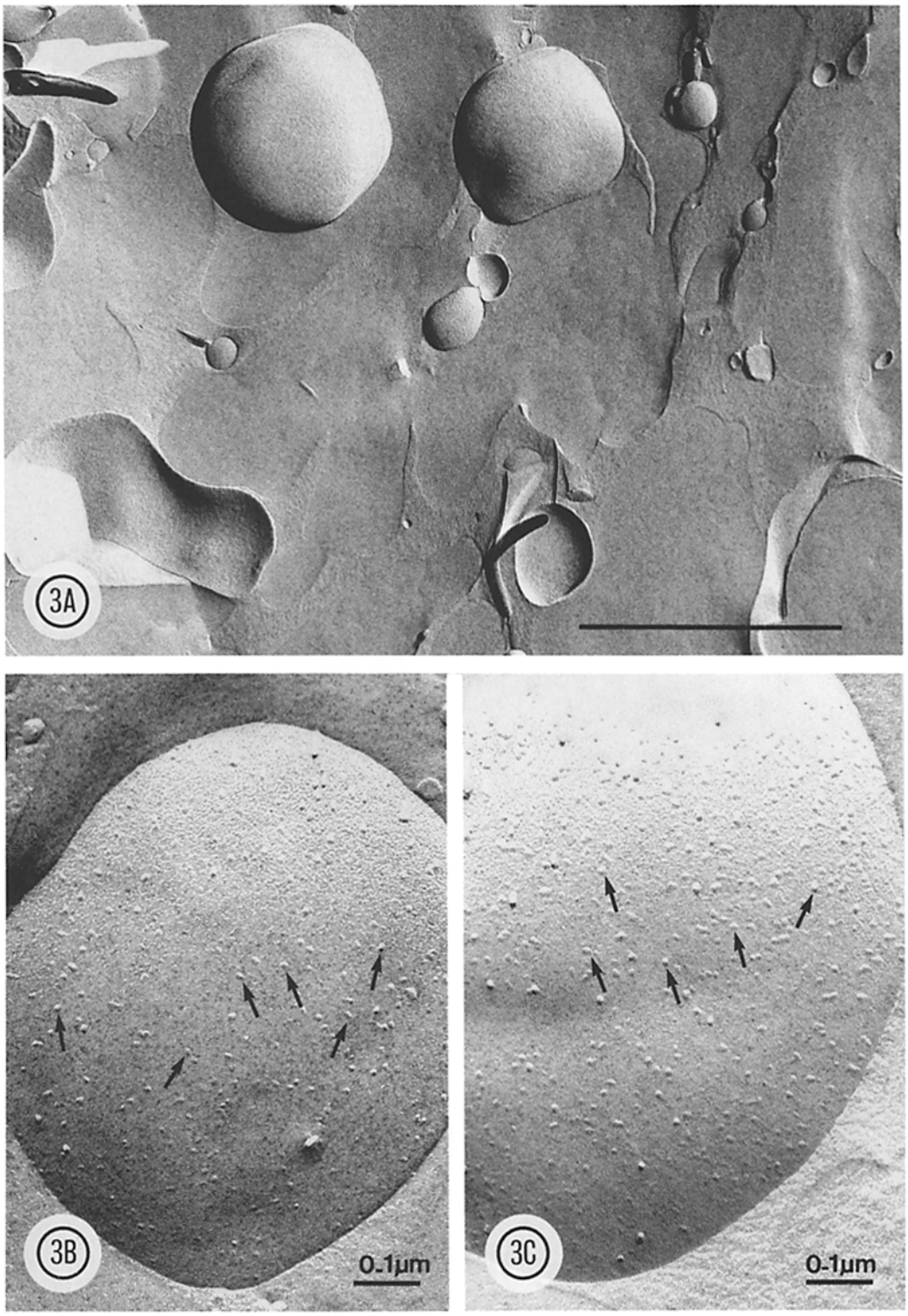

FIGURE 3 Freeze-fracture replicas of the large vesicle preparations with and without membrane proteins. (*A*) Large bilayer vesicles prepared exclusively from phospholipids showing complete absence of membrane particles. Bar, 1 μm. × 40,000. (*B* and *C*) Higher magnifications of large vesicles containing bovine rhodopsin (*B*) or squid rhodopsin (*C*) illustrating that there are numerous particles of ~80Å in diameter (arrows). × 100,000.

lar) for ether extracts is ~1:3 whereas for pentane it is ~1:20. These differences are probably determined by the nature of the emulsion formed and the rate of solvent removal and must be established for the individual system under study.

Electron micrographs of negatively stained and freeze-cleaved replicas of large vesicles prepared from ether extracts of bovine rhodopsin-phospholipid complexes are illustrated in Fig. 2*A* and *B*, respectively. More than 75% of the membrane area is incorporated into vesicles larger than 0.5 μm and ~40% into vesicles larger than 1 μm. More than 90% of the vesicles observed were unilamellar and it was actually exceptional to find multilamellar structures composed of a few concentric bilayers.

Freeze-fracture replicas of the large vesicles prepared, under identical conditions, from exclusively phospholipids (Fig. 3*A*) or from extracts of bovine rhodopsin (Fig. 3*B*) and squid rhodopsin (Fig. 3*C*) are shown in Fig. 3. The most salient difference between the systems is the occurrence of particles ~80 Å in diameter within the fracture faces of the rhodopsin-containing vesicles (Fig. 3*B* and *C*). The freeze-cleaved particles are present in both concave and convex faces and, to a first approximation, appear to be equally distributed. This indicates that rhodopsin is symmetrically incorporated into the large bilayer vesicles, in agreement with the results obtained in the small bilayer vesicles (cf. reference 10).

We have previously reported that rhodopsin-containing planar bilayers display electrical properties consistent with the formation of voltage-sensitive ion channels (16). The planar bilayers are prepared from monolayers of rhodopsin-lipid complexes in solvents; the large vesicles described here are also formed from these extracts. Neither planar bilayers nor vesicles provide all the information necessary for an adequate understanding of the membrane properties. However, we are now in a position to use them in conjunction, enabling us to apply almost any of the available techniques and to attack the rhodopsin problem at different levels of organization of the bilayer system.

In conclusion, a relatively simple procedure to incorporate membrane proteins into large single bilayer vesicles is described. The principles developed are of general use and have already proved to be applicable to four distinct membrane proteins: bovine rhodopsin, squid rhodopsin, reaction centers of *Rhodopseudomonas spheroides*,[1] and acetylcholine receptors from *Torpedo californica*.[2] In addition, it can be readily extended to cytochrome *c* oxidase (14). The large size and encapsulation ability of the vesicles allows the use of the conventional tools and techniques of cell biology and electrophysiology. The potential use of large vesicles as carriers for enzymes, drugs, or macromolecules can be exploited by the incorporation of proteins with chemical determinants for specific surface recognition. This may open new avenues to modify cellular or biochemical processes by taking advantage of those cell surface events involved in the stereo-specific recognition of environmental molecular signals. Current attempts in this direction suggest that this approach might be fruitful (6).

We are indebted to Professor Warren L. Butler for the generous availability of his spectrophotometric facilities.

This work was supported by grants from the National Eye Institute EY-02084 (to M. Montal) and the Muscular Dystrophy Association of America (to M. H. Ellisman).

Received for publication 18 December 1978, and in revised form 9 February 1979.

REFERENCES

1. Antanavage, J., T. F. Chien, Y. C. Ching, L. Dunlap, P. Mueller, and B. Rudy. 1978. Formation and properties of cell-size single bilayer vesicles. *Biophys. J.* **21**:122*a*.
2. Butler, W. L., and D. W. Hopkins. 1970. Higher derivative analysis of complex absorption spectra. *Photochem. Photobiol.* **12**:439-450.
3. Darszon, A., M. Philipp, J. Zarco, and M. Montal. 1978. Rhodopsin-phospholipid complexes in apolar solvents: formation and properties. *J. Membr. Biol.* **43**:71-90.
4. Darszon, A., R. Strasser, and M. Montal. 1978. Photochemistry of rhodopsin-phospholipid complexes in apolar environments. VI International Biophysical Congress (IUPAB). Kyoto, Japan, Abstr. VIII-12-(510). p. 398.
5. Deamer, D. W. 1978. Preparation and properties of ether injection liposomes. *Ann. N. Y. Acad. Sci.* **308**:250-258.
6. Engelhard, V. H., B. C. Guild, A.Helenius, C. Terhorst, and J. L. Strominger. 1978. Reconstitution of purified detergent-soluble HLA-A and HLA-B antigens into phospholipid vesicles. *Proc. Natl. Acad. Sci. U. S. A.* **75**:3230-3234.
7. Fisher, K., and D. Branton. 1974. Application of the freeze-fracture technique to natural membranes. *Methods Enzymol.* **32B**:35-44.
8. Hubbard, R., and R. C. C. St. George. 1958. The rhodopsin system of the squid. *J. Gen. Physiol.* **41**:501-528.
9. Hubbard, R., and G. Wald. 1952. Cis-trans isomers of vitamin A and retinene in the rhodopsin system. *J. Gen. Physiol.* **36**:269-315.
10. Hubbell, W., K.-K. Fung, K. Hong, and Y. S. Chen. 1977. Molecular anatomy and light-dependent processes in photoreceptor membranes. *In* Vertebrate Photoreception. H. Barlow and P. Fatt,

[1] Schöenfeld, M., G. Feher, and M. Montal. Unpublished observations.

[2] Anholt, R., J. Lindstrom, and M. Montal. Unpublished observations.

editors. Academic Press, Inc., New York. 41–59.
11. KAGAWA, Y. 1978. *Biochim. Biophys. Acta.* In press.
12. KAGAWA, Y., and E. RACKER. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation. XXV. Reconstitution of vesicles catalyzing ^{32}Pi-adenosine triphosphate exchange. *J. Biol. Chem.* **246:** 5477–5487.
13. MCCONNELL, D. G. 1965. The isolation of retinal outer segments. *J. Cell Biol.* **27:**459–473.
14. MONTAL, M. 1974. Lipid-protein assembly and the reconstitution of biological membranes. *In* Perspectives in Membrane Biology. S. Estrada and C. Gitler, editors. Academic Press, Inc., New York. 591–622.
15. MONTAL, M. 1976. Experimental membranes and mechanisms of bioenergy transductions. *Annu. Rev. Biophys. Bioeng.* **5:**119–175.
16. MONTAL, M., A. DARSZON, and H. W. TRISSL. 1977. Transmembrane channel formation in rhodopsin-containing bilayer membranes. *Nature Lond.* **267:**221–225.
17. MUELLER, P., and D. O. RUDIN. 1969. Translocators in bimolecular lipid membranes: their role in dissipative and conservative bioenergy transductions. *Curr. Top. Bioenerg.* **3:**157–249.
18. MUNN, E. A. 1974. The application of the negative staining technique to the study of membranes. *Methods Enzymol.* **32B:**20–35.
19. PAPAHADJOPOULOS, D., and W. J. VAIL. 1978. Incorporation of macromolecules within large unilamellar vesicles (LUV). *Ann. N. Y. Acad. Sci.* **308:**259–267.
20. PAPERMASTER, D. S., and W. J. DREYER. 1974. Rhodopsin content in the outer segment membranes of bovine and frog retinal rods. *Biochemistry.* **13:**2438–2444.
21. RACKER, E. 1977. Perspectives and limitations of resolutions-reconstitution experiments. *J. Supramol. Biol.* **6:**215–228.
22. REEVES, J. P., and R. M. DOWBEN. 1969. Formation and properties of thin-walled phospholipid vesicles. *J. Cell Physiol.* **73:**49–60.
23. REEVES, J. P., and R. M. DOWBEN. 1970. Water permeability of phospholipid vesicles. *J. Membr. Biol.* **3:**123–141.
24. STUBBS, G. W., and B. J. LITMAN. 1978. Effect of alterations in the amphipathic microenvironment on the conformational stability of bovine opsin. 1. Mechanism of solubilization of disk membranes by the nonionic detergent, octyl glucoside. *Biochemistry.* **17:**215–219.
25. SZOKA, F., and D. PAPAHADJOPOULOS. 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. U. S. A.* **75:**4194–4198.